

Associations of Fetal Growth Outcomes with Measures of the Combined Xenoestrogenic Activity of Maternal Serum Perfluorinated Alkyl Acids in Danish Pregnant Women

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BACKGROUND: Higher concentrations of single perfluorinated alkyl acids (PFAAs) have been associated with lower birth weight (BW), but few studies have examined the combined effects of PFAA mixtures. PFAAs have been reported to induce estrogen receptor (ER) transactivity, and estrogens may influence human fetal growth. We hypothesize that mixtures of PFAAs may affect human fetal growth by disrupting the ER.

OBJECTIVES: We aimed to study the associations between the combined xenoestrogenic activity of PFAAs in pregnant women's serum and offspring BW, length, and head circumference.

METHODS: We extracted the actual mixture of PFAAs from the serum of 702 Danish pregnant women (gestational wk 11–13) enrolled in the Aarhus Birth Cohort (ABC) using solid phase extraction, high-performance liquid chromatography (HPLC), and weak anion exchange. PFAA-induced xenoestrogenic receptor transactivation (XER) was determined using the stable transfected MVLN cell line. Associations between XER and measures of fetal growth were estimated using multivariable linear regression with primary adjustment for maternal age, body mass index (BMI), educational level, smoking, and alcohol intake, and sensitivity analyses with additional adjustment for gestational age (GA) (linear and quadratic).

RESULTS: On average, an interquartile range (IQR) increase in XER was associated with a 48 g [95% confidence interval (CI): –90, –6] decrease in BW and a 0.3 cm (95% CI: 0.1, 0.5) decrease in birth length. Upon additional adjustment for GA, the estimated mean differences were –28 g (95% CI: –60, 4) and –0.2 cm (95% CI: –0.4, 0.0), respectively.

CONCLUSION: Higher-serum PFAA-induced xenoestrogenic activities were associated with lower BW and length in offspring, suggesting that PFAA mixtures may affect fetal growth by disrupting ER function. <https://doi.org/10.1289/EHP1884>

Introduction

Perfluorinated alkyl acids (PFAAs) are synthetic surfactants used in food packaging, impregnation of, e.g., shoes and textiles and other products. PFAAs are persistent in the environment and bioaccumulate in the food chain (Giesy and Kannan 2002). They are detected in many dietary items including meat, fish, dairy products, cereals, vegetables, and drinking water (Haug et al. 2010; Pérez et al. 2014). Hence, humans are continuously exposed to PFAAs, and the excretion of some PFAA congeners is very slow (Krafft and Riess 2015). Studies of individual PFAA congeners suggest that PFAAs may interfere with the estrogen system *in vitro* (Benninghoff et al. 2011; Henry and Fair 2013; Hu et al. 2003; Kang et al. 2016; Kjeldsen and Bonefeld-Jørgensen 2013; Liu et al. 2007; Rosenmai et al. 2013; Sonthithai et al. 2016), in

fish (Benninghoff et al. 2011; Fang et al. 2012), and in humans (Barrett et al. 2015; Itoh et al. 2016; Knox et al. 2011; Lopez-Espinosa et al. 2016).

A recent systematic review by Bach and coworkers included eight studies of associations between birth weight (BW; continuous) and perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) concentrations in umbilical cord blood or biological samples from pregnant women (Bach et al. 2015). Higher levels of PFOA were associated with lower BW in all of the studies, and six of the eight studies reported a similar association with PFOS, the most abundant PFAA in human serum (Bach et al. 2015). In a recent study of 1,507 pregnant nulliparous women from the Aarhus Birth Cohort (ABC), we found limited evidence of lower BW in association with higher maternal serum PFOS concentrations and higher BW with higher PFOA concentrations (Bach et al. 2016). Associations between serum concentrations of five other PFAAs and BW, birth length, head circumference, and gestational age (GA) were weak, and the direction of the associations varied among the congeners (Bach et al. 2016). Results may vary among different epidemiological studies due to differences in concentrations and combinations of the various PFAAs across study populations.

Human serum contains complex mixtures of PFOS, PFOA, and several other PFAAs, but studies of the combined effects of PFAA mixtures on human health and reproduction have been limited due to the complexity of studying combinations of chemicals. We have developed a method to extract the actual mixture of PFAAs from human serum while simultaneously removing endogenous hormones (Bjerregaard-Olesen et al. 2015). Using this method and an MVLN cell culture assay, we recently analyzed estrogen receptor

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Supplemental Material is available online (<https://doi.org/10.1289/EHP1884>).

The authors declare that they have no actual or potential competing financial interests.

Received 10 March 2017; Revised 20 December 2018; Accepted 24 December 2018; Published 24 January 2019.

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(ER) transactivation induced by PFAA extracts from serum samples provided by 397 pregnant women (Bjerregaard-Olesen et al. 2016d). More than half of the serum extracts agonized the ER transactivation and enhanced the effect of the natural ER ligand 17 β -estradiol (E2) (Bjerregaard-Olesen et al. 2016d).

Epidemiological studies have reported that higher concentrations of estriol (E3) in maternal serum (second trimester) and estetrol (E4) in cord blood are associated with higher BW (Bukowski et al. 2012; Hickey et al. 2014). On the contrary, high concentrations of E2 on the day of human chorionic gonadotropin administration to women undergoing *in vitro* fertilization have been associated with low BW and small-for-gestational-age births (Hu et al. 2014; Pereira et al. 2015). Due to slow excretion and continuous exposure, PFAAs are present in the pregnant women's serum both before conception and during pregnancy. As PFAAs have been found to have xenoestrogenic properties, we hypothesize that PFAAs may suppress human fetal growth through disruption of the ER functions. Birth size is an important indicator of perinatal morbidity that may also predict adverse health outcomes later in life (Alexander et al. 1996). In the present study, we aimed to examine the association between the combined PFAA xenoestrogenic activities of serum PFAA extracts from 702 pregnant women and the BW, birth length, and head circumference of their offspring.

Materials and Methods

Participants and Serum Samples

The present study is a part of the FETOTOX project, which includes pregnant women from five birth cohorts (Bjerregaard-Olesen et al. 2016c). One of the FETOTOX cohorts, the ABC, enrolled women who gave birth at Aarhus University Hospital (Skejby, Denmark) during 2008–2013, with a participation rate of 45–48% (Mortensen et al. 2013). The FETOTOX project included only nulliparous women from the ABC in order to avoid confounding by parity (Bach et al. 2016). In addition, only women who donated a blood sample between gestational wk 9 to 20 and gave birth to a live-born singleton were eligible for the project (Figure 1). We randomly selected 1,533 of the 2,853 women who fulfilled these criteria. We did not exclude children with malformations or other abnormalities. We included 1,507 of these nulliparous women in a previous study of associations between maternal serum PFAA concentrations and indices of fetal growth (Bach et al. 2016). For the present study, we further restricted the study population to women who donated a blood sample before gestational wk 14 to avoid high serum levels of endogenous hormones. In addition, as the toxicological analyses of the FETOTOX project (including the analyses for the present study) are quite time-consuming and expensive, we reduced the number of samples to 702 of the 801 samples collected from women enrolled during 2011–2013 (Figure 1). This group included 397 women randomly selected for a previous study of the xenoestrogenic activities of serum PFAA extracts (Bjerregaard-Olesen et al. 2016d), plus a random sample of 305 additional women.

The blood samples were processed within 2 h after blood draw, and the serum was stored at -80°C (Mortensen et al. 2013). The women filled out questionnaires with information about height, weight, previous miscarriages, educational level, smoking, alcohol intake, and country of birth. GA at birth was determined using first-trimester ultrasound measurements. As described in our recent publication, we identified no implausible values of GA at birth (<24 wk or >45 wk) (Bach et al. 2016). The attending midwives recorded information on the pregnancy outcomes immediately after birth using a structured registration

form. All participants consented to storage of their serum samples in the biobank, and agreed that the serum and information could be used for research.

Serum voluntarily donated to the Aarhus University Hospital Blood Bank by women under 30 y of age was pooled and used for interassay controls, which we refer to as KHK.

The Danish Data Protection Agency (ref. 2011-41-6014) and the Danish National Committee on Health Research Ethics (ref. M-20110054) approved the study.

Methods

Perfluorinated alkyl acids quantification. Sixteen PFAAs [perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), perfluoroheptane sulfonate (PFHpS), PFOS, perfluorodecane sulfonate (PFDS), perfluorooctane sulfonamide (PFOSA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTrA), and perfluorotetradecanoic acid (PFTeA)] were analyzed in the maternal serum using solid phase extraction and liquid chromatography–tandem mass spectrometry as described previously (Bjerregaard-Olesen et al. 2016d; Bonefeld-Jørgensen et al. 2014; Bossi et al. 2005). We used ^{13}C -labeled PFAAs. Seven individual PFAAs (PFHxS, PFOS, PFOA, PFNA, PFDA, PFHpS, and PFUnA) were detected above the limit of quantification (LOQ) in at least 50% of the samples and evaluated in relation to birth outcomes. PFOSA and PFTeA were below the LOQ in all of the samples. The remaining PFAAs were detected in $<50\%$ of the samples. Further technical details, including limits of detection and limits of quantification and percentage above the LOQ, can be found in Table S1.

Perfluorinated alkyl acid extraction prior to estrogen receptor transactivation analysis. PFAAs were extracted from human maternal serum while simultaneously removing endogenous hormones such as estrone, E2, and testosterone as previously described (Bjerregaard-Olesen et al. 2015). Briefly, 3 mL serum was extracted by solid phase extraction on an OASIS HLB cartridge (6 mL, 500 cc; Waters) with elution using 4 mL methanol and 4 mL ethyl acetate. The eluates were concentrated by vacuum centrifugation, extracted two times with *n*-hexane:ethyl acetate (9:1) and two times with tetrahydrofuran:*n*-hexane (3:2). The supernatants from the latter extraction were evaporated at 30°C under N_2 until near dryness. Upon reconstitution in 315 μL tetrahydrofuran:*n*-hexane (4:1), the extracts were fractionated by high-performance liquid chromatography (HPLC) on an Alliance 2695 separation module (Waters) equipped with a normal-phase Spherisorb Si60 analytical column (250×4.6 mm ID, 5 μm ; Waters). The HPLC fractionation was run with a constant flow rate of 1.5 mL/min, using two eluents: A) *n*-hexane, and B) *n*-hexane:isopropanol:methanol (40:15:45; vol:vol:vol). The gradient elution was initiated with 10% B for 18 min, changing linearly to 50% B over 4 min, followed by a 13-min linear gradient back to 10% B, which was maintained for the final 5 min of the total 40-min run. The eluate was collected in several fractions with the PFAAs in fraction F3 (collected between 22 and 26 min). Fraction F3 was further extracted by weak anion exchange on an OASIS WAX cartridge (6 mL, 150 mg, 30 μm ; Waters). Neutral compounds such as E3 and E4 were eluted using 4 mL methanol in a fraction referred to as F3-W1, which was collected but not used in this study. The PFAAs were eluted using 0.1% ammonium hydroxide in methanol in a fraction referred to as F3-W2. The PFAA fraction F3-W2 was evaporated by vacuum centrifugation, and the dry fractions were stored at -80°C .

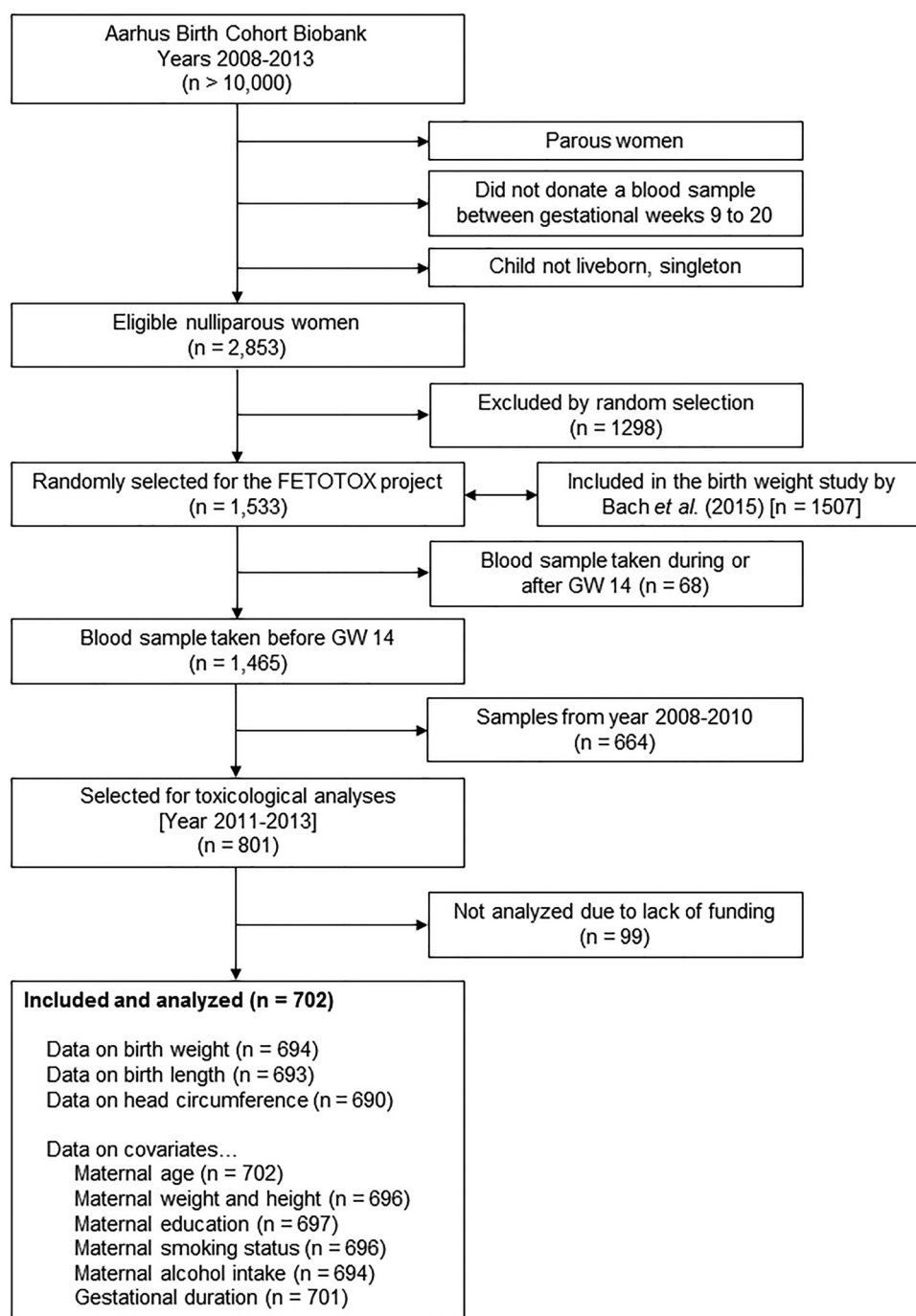


Figure 1. Flowchart for inclusion of study participants. Note: GW, gestational wk.

Each extraction batch consisted of 22 randomly selected serum samples, one KHK serum control, and one procedural blank consisting of double-distilled water. The KHK was used to secure robustness between the assays. The procedural blank was used to control for potential PFAA contamination during the extraction, as some laboratory materials may contain PFAAs (van Leeuwen and de Boer 2007).

Estrogen receptor transactivation luciferase reporter gene assay. ER transactivation was analyzed using the stable transfected MVLN cell line (provided by M. Pons, France) carrying the estrogen response element luciferase reporter vector (Demirpence et al. 1993; Pons et al. 1990) as described previously (Bonefeld-Jorgensen et al. 2005; Hjelmberg et al. 2006). Briefly, 8.5×10^4

cells were seeded in each well in a 96-well plate and left at 37°C in the incubator overnight. The next day, the dry PFAA fractions (F3-W2) were reconstituted in 20 μ L EtOH:H₂O:dimethylsulfoxide (50:40:10, vol/vol/vol) and 200 μ L of Dulbecco's modified Eagle's media without phenol red (DMEM; Lonza) containing 0.5% charcoal–dextran stripped fetal bovine serum. These solutions were then divided into two portions of 100 μ L each, which were diluted with 400 μ L DMEM media (i.e., noncompetitive design) or 400 μ L DMEM media containing 30 picomolar (pM) E2 (i.e., competitive design with a final E2 concentration of 24 pM). The reconstituted PFAA fractions (F3-W2) with and without E2 coexposure were added to the 96-well plate in triplicate, using 100 μ L/well. After 24 h incubation at 37°C, the cells were harvested, the luciferase activity

was determined in a LUMIstar luminometer (BMG Labtech, RAMCON), and the protein content was determined by fluorometric measurements using a WALLAC Victor2 (Perkin Elmer). The ER transactivation data were expressed as relative light units per count of protein. To avoid mutations, we discarded the cells after a maximum of 10 passages.

As controls of the comparability between assays, we analyzed 25 pM E2 (positive control), the F3-W2 fraction from a KHK serum control, and a procedural blank in parallel in each assay. The ER transactivation interassay coefficient of variation of the procedural blank relative to the DMEM media and positive control (i.e., 25 pM E2) was 15.6% in the noncompetitive assays and 11.5% in the competitive assays.

Exposure variables. The xenoestrogenic receptor transactivation (XER) for the extracts alone in the noncompetitive assay (XER) and upon coexposure with E2 in the competitive assay (XERcomp) was expressed as a percentage of the procedural blank \pm E2 (set to 100%). The XER is a measure of the xenoestrogenic activity elicited by the PFAA fraction F3-W2 alone. The XERcomp is a measure of the competitive/combinatory xenoestrogenic effects elicited by the PFAA fraction F3-W2 combined with a physiologically relevant concentration (24 pM) of the natural estrogen E2. The variation was below 10% between the triplicate XER and XERcomp measurements of each sample for 88 and 92% of the samples, respectively, but we allowed up to 25% variation.

In addition to the XER and XERcomp, we also calculated the estradiol equivalent for the F3-W2 fractions (W2-EEQ; Figure S1). The W2-EEQ is a measure of the concentration of E2 needed to elicit an effect similar to what was elicited by the PFAA fraction F3-W2. The calculation was done by interpolation from the ER transactivation using a sigmoidal E2 concentration–transactivation curve (concentration range: 1.5–300 pM; Figure S1), which was analyzed in parallel with the F3-W2 fractions on a separate 96-well plate in each assay. PFAA fractions eliciting an ER transactivation higher than the upper LOQ (ULOQ) were assigned W2-EEQ values of 150 pM (the median of the E2 concentrations that produced a maximal effect). PFAA fractions eliciting an ER transactivation below the lower LOQ (LLOQ) were assigned W2-EEQ values of 0.07 pM (the lowest W2-EEQ value above the LLOQ across the assays divided by the square root of 2). The W2-EEQ values that were negative upon subtracting the procedural blank were replaced with W2-EEQ values of 0.05 pM (the lowest W2-EEQ value above the LLOQ divided by 2). A quantifiable and positive W2-EEQ was found for 60% of the PFAA fractions, whereas 0.4% were above the ULOQ, 24% were below the LLOQ, and 16% were negative upon subtracting the blank.

Outcome variables. The following indices of fetal growth were included as outcome variables in the study: BW, birth length, and head circumference. Due to missing data, a few participants were excluded from the analyses involving BW ($n=8$), birth length ($n=9$), and head circumference ($n=12$). One implausible combination of BW and GA was identified (Alexander et al. 1996), such as BW 4,200 g and GA 24 wk, and this participant was excluded from the analyses involving GA.

Statistical Analyses

The statistical analyses were performed using STATA/IC (version 13; StataCorp), Microsoft Excel, and SigmaPlot (version 11.0; Systat Software).

\sum PFCA was calculated by summing the concentrations of the 10 analyzed perfluorinated carboxylates (PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, and

PFTeA). \sum PFSA was calculated by summing the concentrations of the six analyzed perfluorinated sulfonates (PFBS, PFHxS, PFHpS, PFOS, PFDS, and PFOSA). \sum PFAA was calculated by summing the concentrations of all 16 analyzed PFAAs (PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, PFBS, PFHxS, PFHpS, PFOS, PFDS, and PFOSA). Only individual PFAAs being detected in at least 50% of the samples $>$ LOQ (PFHxS, PFOS, PFOA, PFNA, PFDA, PFHpS, and PFUnA) were evaluated in relation to birth outcomes, and \sum PFAAs were not included along the analyses of individual compounds since only the relation to single compounds were of interest.

A principal component analysis (PCA) was used to group the correlated PFAAs into factors. The number of factors was determined using a scree plot after an orthogonal rotation. Factor loadings higher than 0.4 or lower than -0.4 were considered important. Pearson's correlation analysis and linear regression analysis were used to identify associations between ln-transformed PFAA concentrations, PCA factors, and ln-transformed xenoestrogenic activities (XER, XERcomp, and W2-EEQ). Only the PFAAs detected in $>50\%$ of the samples were included in the statistical analyses. Samples $<$ LOQ were excluded from all statistical analysis and also from the PCA analyses. PFAAs that were $<$ LOQ in $\geq 50\%$ of samples were still included in the summed PFAA concentration. For concentrations $<$ LOQ for the seven individual PFAAs that were evaluated in relation to the birth outcomes, we used a concentration of LOQ/2. The associations between interquartile ranges (IQRs) of PFAA concentrations or xenoestrogenic activities and BW, birth length, and head circumference were investigated using multivariable linear regression with robust standard errors (Huber-White sandwich estimator). We performed complete-case analyses that excluded observations with missing covariate data. In the primary analysis (Model 1), we adjusted for the covariates identified *a priori* using directed acyclic graphs (Figures S2, S3, and S4) as follows. Maternal age (continuous), maternal prepregnancy body mass index [BMI (four categories)], maternal level of education (four categories), maternal smoking (three categories), and maternal alcohol intake (three categories). In addition, we performed two sensitivity analyses: Model 2 was adjusted for the Model 1 covariates plus gestational duration (linear and quadratic); Model 3 included Model 1 covariates and was restricted to term births (gestational duration above 37 wk and 0 d; total $n=633$) (Figure S2).

In addition to the continuous analysis, the associations between the xenoestrogenic activities and pregnancy outcomes were also investigated on a categorical scale. Thus, we divided the XER and XERcomp variables into quartiles. Because more than 40% of W2-EEQ values were $<$ LLOQ or negative after subtracting the procedural blank, the W2-EEQ values were categorized, such as 0.05–0.07 pM (samples $<$ LLOQ or negative after subtracting the procedural blank, 40%, $n=278$), >0.07 –1.5 pM (where 1.5 pM was median, 10%, $n=73$), >1.5 –9.7 pM (25%, $n=177$), and >9.7 –150 pM (where 150 pM was the value assigned to samples $>$ ULOQ, 25%, $n=174$) (see also Figure S1). Higher quartiles/categories were compared to the lowest quartile/category (reference) using multivariable linear regression. We adjusted for the same covariates as described above for the continuous analyses.

Analyses of associations between the xenoestrogenic activities and indices of fetal growth were performed separately for each sex as well as pooled, because previous reports have found sex differences concerning the associations between PFAA concentrations and indices of fetal growth (Andersen et al. 2010; Bach et al. 2016; Maisonet et al. 2012; Robledo et al. 2015; Washino et al. 2009).

Results

The median values of BW, birth length, and head circumference of the newborns were 3,460 g (25th, 75th percentiles: 3,180; 3,770), 52 cm (25th, 75th percentiles: 50, 53), and 35 cm (25th, 75th percentiles: 34, 36), respectively (Table S2). The median maternal age at delivery was 29 y of age, and median GA at birth was 40 wk (Table S2).

Table 1 presents the baseline characteristics of the 702 pregnant women, including median xenoestrogenic activities and \sum PFAA concentrations within each category of the women. Most women were nonsmokers (87%) and had a prepregnancy BMI between 18.5 and 24.9 kg/m² (73%). There were few women with a low educational level (3%), but the other education categories were well represented (28–38%). Eleven percent of the women consumed alcohol during pregnancy. Few observations were missing covariate data (Table 1).

Compared with other women, median XER values were lowest for underweight women (BMI < 18.5 kg/m²) and women with a lower middle educational level and highest for women with an upper middle or high educational level, women who smoked until pregnancy, and women who only drank alcohol before pregnancy (Table 1). The median XERcomp was highest for underweight women (BMI < 18.5 kg/m²) and women who drank alcohol during pregnancy, and lowest for women with a low educational level. The W2-EEQ was lowest for overweight women (BMI: 25–30 kg/m²), women who smoked during pregnancy, and women who never drank alcohol, and highest for obese women (BMI > 30 kg/m²), women with a low educational level, and women who only smoked before pregnancy (Table 1).

The women in the present study were selected from a larger population included in a previous study (Bach et al. 2016). The median \sum PFAA concentration was lower in the present study (12.7 vs. 14.3 ng/mL), and more women reported that they never drank alcohol compared with the previous study population (43 vs. 33%) (Table S2).

PFAA concentrations and xenoestrogenic activities positively correlated between all of the individual PFAAs except for PFHxS and XER (Figure 2 and Table S3). The \sum PFCA and \sum PFSA elicited the highest correlation to carboxylated and sulfonated compounds, respectively (Figure 2). In addition, \sum PFCA, \sum PFSA, and \sum PFAA correlated positively to the xenoestrogenic activities (Table S3). Using PCA, three factors were identified (Figure 3). The proportion of variability was 0.42 for Factor 1, 0.35 for Factor 2, and 0.31 for Factor 3 (Figure 3). Factor 1 had high positive loadings of PFNA, PFOA, PFDA, and PFOS (Figure 3). Factor 2 had high positive loadings of the sulfonates: PFHpS, PFOS, and PFHxS, and Factor 3 had high positive loadings of PFUnA, PFDA, and PFNA (Figure 3). Although the correlation coefficients were low ($r < 0.11$), Factor 1 was positively associated with all of the xenoestrogenic activity variables, significant for XERcomp and W2-EEQ (Figure 3). Factor 2 had a nonsignificant inverse association with XER and nonsignificant positive associations with XERcomp and W2-EEQ. Factor 3 was positively associated with all three activity variables, with a significant association with XER (Figure 3). Seven individual PFAAs (PFHxS, PFOS, PFOA, PFNA, PFDA, PFHpS, and PFUnA) were detected > LOQ in at least 50% of the samples and evaluated in relation to birth outcomes. PFOSA and PFTeA were below the limit of quantification

Table 1. Characteristics of the 702 nulliparous pregnant women included in the study population and median values of the xenoestrogenic activity variables and \sum PFAA, Aarhus Birth Cohort 2011–2013.

Characteristic	n (%)	XER	XERcomp	W2-EEQ (pM)	\sum PFAA (ng/mL)
All participants	702 (100%)	123.8	111.1	1.5	12.7
Prepregnancy BMI	—	—	—	—	—
< 18.5 kg/m ²	24 (3%)	117.1	123.5	1.1	13.7
18.5–25 kg/m ²	514 (74%)	123.0	110.7	1.6	12.9
25–30 kg/m ²	101 (15%)	124.9	109.8	0.7	12.1
> 30 kg/m ²	57 (8%)	120.8	112.3	2.0	13.1
Missing	6	—	—	—	—
Education	—	—	—	—	—
Low	18 (3%)	128.3	101.6	3.3	9.3
Lower middle	198 (28%)	117.7	111.3	1.8	12.4
Upper middle	266 (38%)	126.6	112.6	1.4	13.1
High	215 (31%)	126.0	109.4	1.4	13.1
Missing	5	—	—	—	—
Smoking	—	—	—	—	—
Nonsmoker	609 (88%)	123.8	111.3	1.5	12.9
Until pregnancy	66 (9%)	125.8	112.7	2.3	12.4
During pregnancy	21 (3%)	123.8	108.9	0.5	10.2
Missing	6	—	—	—	—
Alcohol	—	—	—	—	—
Never drinker	296 (43%)	121.1	109.4	0.9	12.7
Before pregnancy	322 (46%)	126.1	111.5	1.9	12.6
During pregnancy	76 (11%)	122.0	117.4	1.7	13.4
Missing	8	—	—	—	—
Sex of the child	—	—	—	—	—
Girls	350 (50%)	123.8	111.1	2.0	12.5
Boys	352 (50%)	124.1	111.2	1.3	13.1
Gestational duration	—	—	—	—	—
Term births	660 (94%)	123.6	111.3	1.4	12.7
Preterm births	41 (6%)	135.2	108.8	4.6	12.6
Missing	1	—	—	—	—

Note: —, data not available; BMI, body mass index; W2-EEQ, estrogen equivalence for the serum PFAA fraction F3-W2 alone; XER, xenoestrogenic receptor transactivation in the noncompetitive assay (serum PFAA fractions alone); XERcomp, xenoestrogenic receptor transactivation in the competitive assay (serum PFAA fractions + 24 pM E2); \sum PFAA, summed concentration of 16 perfluorinated alkyl acids [perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), perfluoroheptane sulfonate (PFHpS), perfluorooctane-sulfonic acid (PFOS), perfluorodecane sulfonate (PFDS), perfluorooctane sulfonamide (PFOSA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDDA), perfluorotridecanoic acid (PFTeA), and perfluorotetradecanoic acid (PFTeA)].

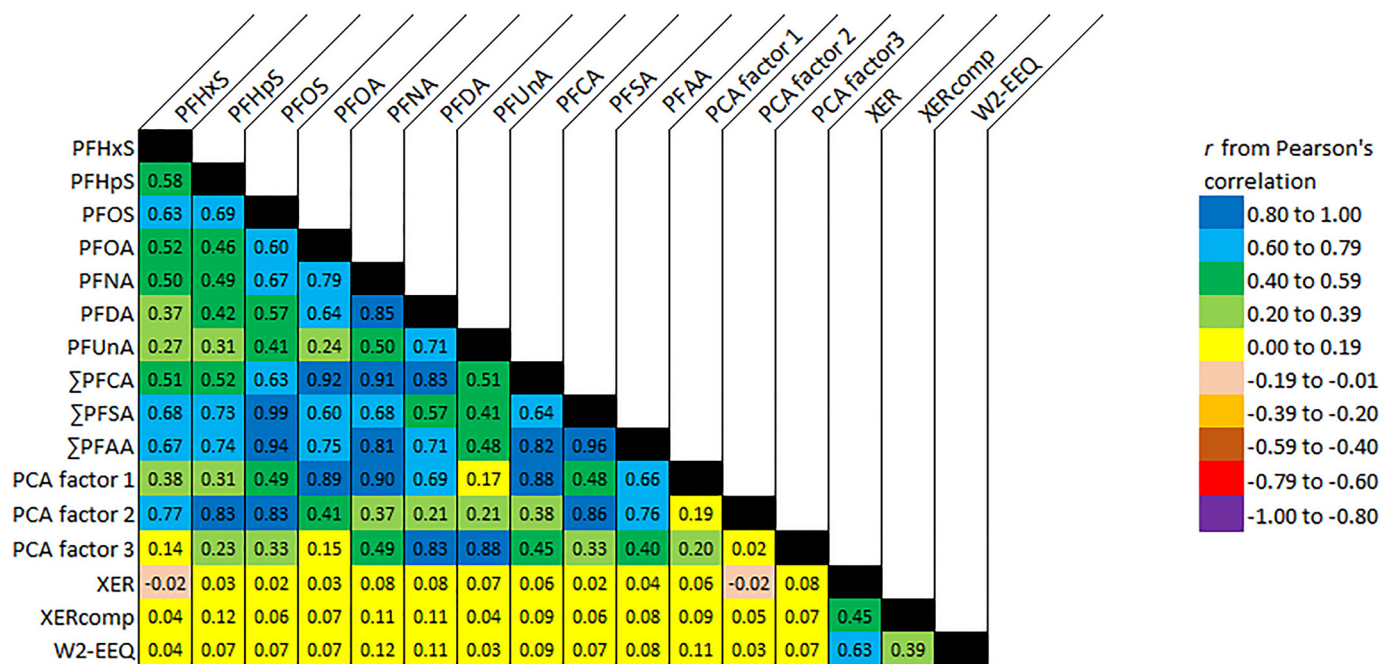


Figure 2. Heatmap with pairwise Pearson's r correlation coefficients for individual and summed serum PFAA concentrations, PCA Factors 1–3, and xenoestrogenic activity variables ($n = 702$). Note: PCA, principal component analysis; W2-EEQ, estrogen equivalence for the serum PFAA fraction F3-W2 alone; XER, xenoestrogenic receptor transactivation in the noncompetitive assay (serum PFAA fractions alone); XERcomp, xenoestrogenic receptor transactivation in the competitive assay (serum PFAA fractions + 24 pM E2); Σ PFAA, sum of the perfluorinated alkyl acids; Σ PFCA, sum of the perfluorinated carboxylic acids [perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTeA), and perfluorotetradecanoic acid (PFTeA)]; Σ PFSA, sum of the perfluorinated sulfonates [perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), perfluoroheptane sulfonate (PFHpS), perfluorooctanesulfonic acid (PFOS), perfluorodecane sulfonate (PFDS), perfluorooctane sulfonamide (PFOSA)].

(LOQ) in all of the samples. The remaining PFAAs were detected in less than 50% of the samples (see “Methods and Materials” section). For single compounds in the primary analysis (Model 1), IQR increases in serum concentrations of PFHpS, PFOS, and PFUnA were associated with lower mean BWs, IQR increases in PFHxS and PFOA were associated with higher mean BWs, and estimates for the difference in BW with IQR increases in PFNA, PFDA, and Σ PFAA were close to the null (Table 2). After further adjustment for gestational duration (Model 2), there was little change in estimates for PFHpS and PFOS, while the inverse association with PFUnA increased from -17 g [95% confidence interval (CI): -52 , 18] to -27 g (95% CI: -53 , -2), and positive associations with PFHxS and PFOA moved closer to the null. The estimated difference in mean BW with an IQR increase in Σ PFAA shifted from -4 g (95% CI: -52 , 45) to -11 g (95% CI: -48 , 27) (Table 2). For the combined xenoestrogenic activity, in the primary analysis (Model 1), IQR increases in XER activity in the PFAA serum fraction were associated with lower BW (-48 g; 95% CI: -90 , -6) and shorter birth length (-0.3 cm; 95% CI: -0.5 , -0.1). Associations with both outcomes were closer to the null but still negative after further adjustment for GA (Model 2) and restricting the model to term births (Model 3). Associations of XER quartiles with BW and length were strongest for the highest vs. lowest quartile comparisons, although associations were not monotonic by quartile (Table 3). IQR increases in W2-EEQ also were associated with lower BW and length (Model 1 estimates of -24 g; 95% CI: -45 , -2 , and -0.1 cm; 95% CI: -0.2 , 0.0 , respectively) (Table 3). Associations with BW were negative but closer to the null after adjustment for GA or restriction to term births, while associations with birth length were similar for Models 1–3. Associations of BW and length with categories of W2-EEQ were nonmonotonic but strongest for the

highest vs. lowest category comparisons (Table 3). XERcomp activity was not associated with BW or length, and associations with head circumference were null for all xenoestrogenic activity variables and models, with the exception of the Model 1 estimate for an IQR increase in XER (-0.1 cm; 95% CI: -0.2 , 0.0) (Table 3).

There were no significant differences between boys and girls in associations between sex-specific IQR increases in the xenoestrogenic activities and pregnancy outcomes (Table 4). However, the association between XERcomp and BW was inverse in boys (Model 1: -16 g; 95% CI: -72 , 39) and positive in girls (23 g; 95% CI: -47 , 93 ; interaction p -value = 0.38) (Table 4).

IQR increases for Σ PFAA, and for five of the seven individual PFAAs, were associated with lower BW in girls and higher BW in boys, resulting in significant differences by sex for PFOS, PFNA, and Σ PFAA (Model 1 interaction p -values ≤ 0.013 and < 0.015 for PFOS and PFNA, and interaction p -value < 0.008 for Σ PFAA) (Table S4). Exceptions to the overall pattern were evident for PFHxS (positive association in boys, positive but close to the null in girls), and PFHpS and PFUnA (inverse associations for boys and girls, although closer to the null for boys). Associations with birth length and head circumference followed a similar pattern, with positive or null estimates for boys and inverse or null estimates for girls, and significant gender differences in birth length for PFOS, PFOA, PFNA, and Σ PFAA (Model 1 interaction p -values ≤ 0.02), and significant differences in head circumference for PFOA and PFNA (Model 1 interaction p -values ≤ 0.004). Patterns of associations by sex and outcome were similar for estimates from Models 2 and 3 (Table S4).

Discussion

In a cohort of Danish pregnant women, we found associations between higher xenoestrogenic activities (XER and W2-EEQ)

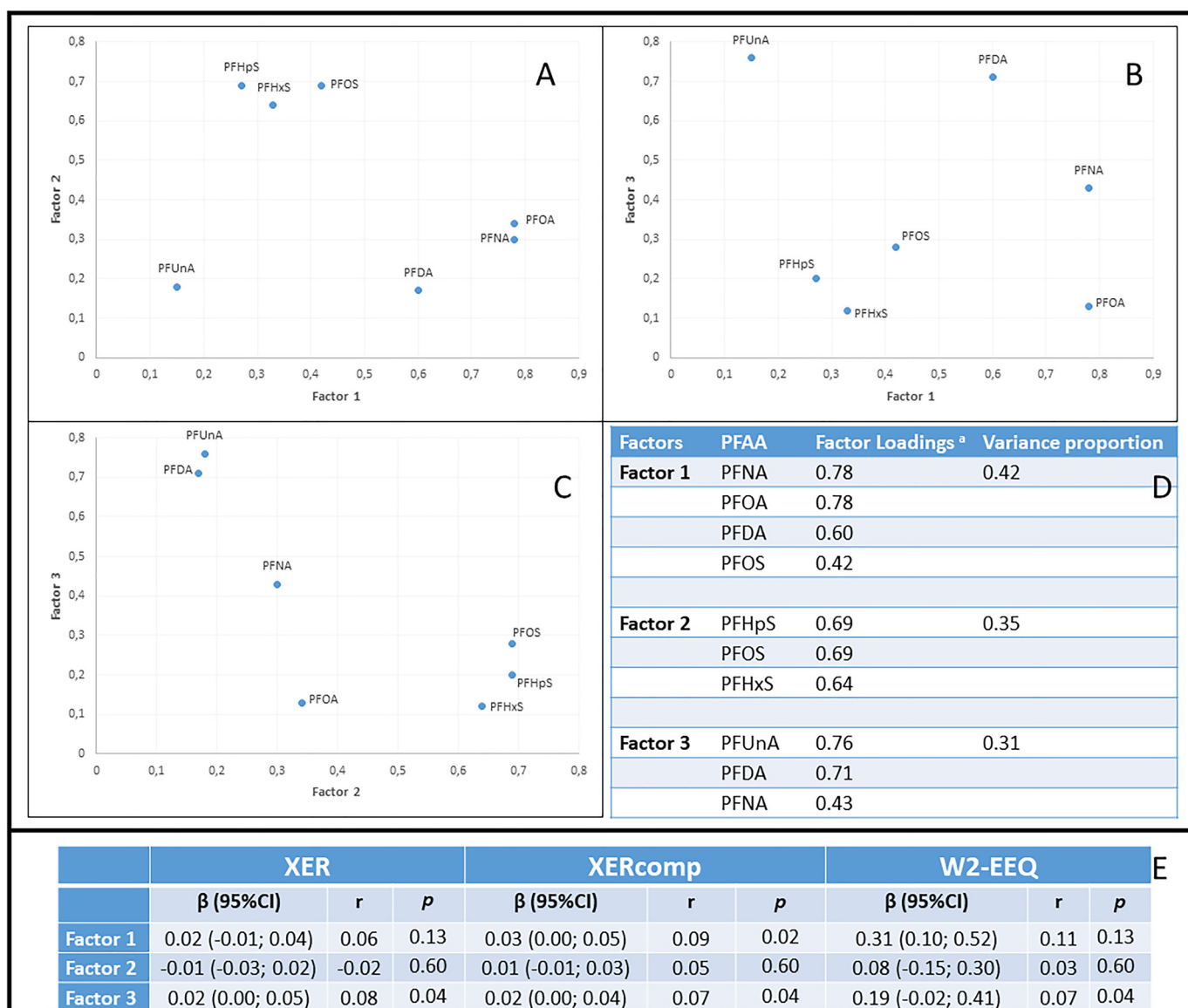


Figure 3. Principal component analysis. (A–D) Rotated factor loadings for ln-transformed perfluorinated alkyl acids (PFAA) concentrations. (E): Linear regression analysis of associations between the principal component factors and ln-transformed xenoestrogenic activities for the PFAA serum fraction in 702 pregnant Danish women. Factor loadings are the correlation coefficients (r) between the PFAA variables and the factors, as given in the x - and y -axes. PFAAs with factor loadings below ± 0.4 are not listed in the table.

induced by serum PFAA extracts and lower BW and shorter birth length in the offspring. The PFAA extracts were obtained using our recently developed method to analyze the xenoestrogenic activity of PFAA mixtures at physiologically relevant concentrations and compositions, acquired by extracting PFAAs from human serum followed by analysis of the ER transactivation induced by the PFAA extracts (F3-W2) in an MVLN cell culture assay (Bjerregaard-Olesen et al. 2015).

We recently reported that serum PFAA concentrations from 397 of the 701 pregnant women included in the present analysis were associated with higher xenoestrogenic activities in the PFAA fractions F3-W2 (Bjerregaard-Olesen et al. 2016d). This finding was corroborated in the present study, which includes an additional 305 women. Three PCA factors based on individual serum PFAA concentrations explained similar proportions of variability, with 0.41 for Factor 1, 0.35 for Factor 2, and 0.31 for Factor 3. XERcomp and W2-EEQ were associated mostly with Factor 1, which was influenced mainly by the perfluorinated

carboxylates PFNA, PFOA, and PFDA plus the perfluorinated sulfonate PFOS, whereas XER was most convincingly associated with Factor 3, which was primarily influenced by PFUnA, PFDA, and PFNA (all perfluorinated carboxylates). Overall, this suggests that the complex mixture of PFAAs, rather than the concentrations of individual PFAAs, determined the ER transactivation. Factor 2, which had high factor loadings for the three perfluorinated sulfonates (PFHpS, PFOS, and PFHxS), was weakly associated with the xenoestrogenic activity variables, which suggests that these PFAAs may have had less influence on ER transactivation than the perfluorinated carboxylates. An *in vitro* study using HEK-293T cells showed that the human ER α was induced by PFNA, PFOA, PFDA, and PFOS (Benninghoff et al. 2011), the PFAAs that had high loadings on our PCA Factor 1. However, our previous *in vitro* study showed that ER transactivation in MVLN cells was induced by PFOS, PFOA, and PFHxS, but not PFNA, PFDA, or PFUnA (Kjeldsen and Bonefeld-Jørgensen 2013). To our knowledge, no study has

Table 2. Regression coefficients [(95% confidence interval (CI)] for fetal growth indices per interquartile range (IQR) increase in serum PFAA concentrations in pregnant women, Aarhus Birth Cohort, 2011–2013.

	Crude <i>n</i> = 694	Model 1 ^a <i>n</i> = 671	Model 2 ^b <i>n</i> = 670	Model 3 ^c <i>n</i> = 633
Birth weight (g)	—	—	—	—
PFHxS	8 (–9, 25)	9 (–7, 25)	5 (–9, 19)	9 (–5, 22)
PFHpS	–27 (–81, 27)	–30 (–85, 25)	–34 (–78, 11)	–33 (–87, 21)
PFOS	–10 (–57, 37)	–15 (–62, 32)	–17 (–55, 22)	–17 (–60, 26)
PFOA	19 (–6, 45)	18 (–9, 45)	9 (–8, 26)	18 (–3, 38)
PFNA	4 (–32, 40)	2 (–35, 38)	0 (–25, 25)	6 (–25, 37)
PFDA	2 (–28, 32)	2 (–29, 33)	–5 (–25, 15)	7 (–17, 31)
PFUnA	–17 (–52, 17)	–17 (–52, 18)	–27 (–53, –2)	–13 (–42, 16)
∑ PFAA	0 (–48, 49)	–4 (–52, 45)	–11 (–48, 27)	–4 (–47, 39)
Birth length (cm)	—	—	—	—
PFHxS	0.1 (0.0, 0.2)	0.1 (0.0, 0.2)	0.1 (0.0, 0.1)	0.1 (0.0, 0.2)
PFHpS	–0.1 (–0.4, 0.2)	–0.1 (–0.4, 0.1)	–0.2 (–0.4, 0.1)	–0.1 (–0.4, 0.1)
PFOS	0.0 (–0.3, 0.2)	–0.1 (–0.3, 0.2)	–0.1 (–0.3, 0.1)	–0.1 (–0.3, 0.2)
PFOA	0.1 (–0.1, 0.2)	0.1 (–0.1, 0.2)	0.0 (–0.1, 0.1)	0.1 (–0.1, 0.2)
PFNA	0.0 (–0.2, 0.2)	0.0 (–0.2, 0.2)	0.0 (–0.2, 0.1)	0.0 (–0.2, 0.2)
PFDA	0.0 (–0.1, 0.2)	0.0 (–0.1, 0.2)	0.0 (–0.1, 0.1)	0.1 (–0.1, 0.2)
PFUnA	0.0 (–0.2, 0.2)	0.0 (–0.2, 0.2)	0.0 (–0.2, 0.1)	0.0 (–0.1, 0.2)
∑ PFAA	0.0 (–0.2, 0.3)	0.0 (–0.3, 0.3)	0.0 (–0.2, 0.2)	0.0 (–0.2, 0.2)
Head circumference (cm)	—	—	—	—
PFHxS	0.0 (–0.1, 0.1)	0.0 (–0.1, 0.1)	0.0 (–0.1, 0.1)	0.0 (–0.1, 0.1)
PFHpS	0.0 (–0.1, 0.2)	0.0 (–0.2, 0.2)	0.0 (–0.2, 0.1)	0.0 (–0.2, 0.2)
PFOS	0.0 (–0.2, 0.1)	0.0 (–0.2, 0.1)	–0.1 (–0.2, 0.1)	–0.1 (–0.2, 0.1)
PFOA	0.1 (0.0, 0.2)	0.1 (0.0, 0.2)	0.1 (0.0, 0.2)	0.1 (0.0, 0.2)
PFNA	0.0 (–0.1, 0.2)	0.0 (–0.1, 0.2)	0.0 (–0.1, 0.2)	0.0 (–0.1, 0.2)
PFDA	0.0 (–0.1, 0.2)	0.0 (–0.1, 0.2)	0.0 (–0.1, 0.1)	0.0 (–0.1, 0.2)
PFUnA	0.0 (–0.2, 0.1)	0.0 (–0.2, 0.1)	0.0 (–0.2, 0.1)	0.0 (–0.1, 0.1)
∑ PFAA	0.0 (–0.2, 0.2)	0.0 (–0.2, 0.2)	0.0 (–0.1, 0.1)	0.0 (–0.2, 0.2)

Note: Interquartile ranges (ng/mL): PFHxS = 0.22; PFHpS = 0.12; PFOS = 4.12; PFOA = 0.92; PFNA = 0.34; PFDA = 0.16; PFUnA = 0.22; ∑ PFAA = 5.65 PFDA, perfluorodecanoic acid; —, data not available; PFHpS, perfluoroheptane sulfonate; PFHxS, perfluorohexane sulfonate; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFOS, perfluorooctanesulfonic acid; PFUnA, perfluoroundecanoic acid; ∑ PFAA, sum of the perfluorinated alkyl acids.

^aModel 1 adjusted for age at delivery (continuous), prepregnancy body mass index (four categories), educational level (four categories), smoking (three categories), and alcohol intake (three categories).

^bModel 2 includes Model 1 covariates plus gestational age at birth (linear and quadratic).

^cModel 3 is restricted to term births (>37 gestational wk and 0 d) and adjusted for Model 1 covariates.

compared ER transactivation induced by perfluorinated sulfonate mixtures with ER transactivation induced by perfluorinated carboxylate mixtures.

In the present study (Model 1), we estimated a mean difference in BW of –48 g (95% CI: –90, –6) per IQR increase in XER, –24 g (95% CI: –45, –2) for W2-EEQ, and –2 g (95% CI: –48, 43) for XERcomp (Table 3). The larger difference in mean BW with IQR increases in XER and W2-EEQ compared with IQR increases in PFAA serum concentrations suggests that, at least for potential effects on birth outcomes, the net effect of the mixture of serum PFAAs on xenoestrogenic activity may be a more etiologically relevant measure of exposure than serum concentrations of individual PFAAs. XER and XERcomp refer to the combined xenoestrogenic transactivity of serum PFAA serum extracts alone and upon addition of 25 pM E2, respectively. The weak associations between XERcomp and birth outcomes suggests that associations between XER and the same outcomes were not a consequence of synergistic or inhibitory effects of serum PFAA mixtures on the natural estrogen E2.

Several studies have reported inverse associations between concentrations of individual PFAAs and BW, as reviewed by Bach et al. (2015). However, it is difficult to compare effect estimates for measures of the combined effects of serum PFAA mixtures on xenoestrogenic activity with effect estimates for individual serum PFAA concentrations because the measures have different end points, such as combined mechanistic effect on ER function and simply concentration vs. BW in grams. A study of residents of the Mid-Ohio Valley during 2005–2010 reported a mean decrease in BW of 5 g (95% CI: –13, 2) in association with a 21.8 ng/mL (IQR) increase in maternal serum

PFOA and 23 g decrease (95% CI: –48, 3) with a 10.2 ng/mL (IQR) increase in maternal serum PFOS (Darrow et al. 2013). In contrast, we estimated that mean BW decreased by 15 g (95% CI: –62, 32) with a 4.12 ng/mL increase in PFOS and increased by 18 g (95% CI: –9, 45) with a 0.92 ng/mL (IQR) increase in maternal serum PFOA. The differences between the studies may reflect population differences in the mixtures of PFAAs, and thus their net xenoestrogenic effects, but other factors, such as differences in susceptibility and unmeasured confounders, could also play a role. A study from Baltimore (2004–2005) with IQR exposure contrasts reported a mean BW decrease of 58 g (95% CI: –119, 3) with a 0.9 ng/mL increase in cord blood PFOA, and a 58 g decrease (95% CI: –125, 9) with a 4.5 ng/mL increase in cord blood PFOS (Apelberg et al. 2007). These associations with individual PFAAs in cord blood were not significantly associated with BW in contrast to a significant inverse association between IQR increase in maternal serum PFAA-induced XER and BW in our study population (Model 1: –48 g; 95% CI: –90, –6). This may be at least partly due to the use of different biological samples, as the ratio between maternal serum and cord blood concentrations is not 1:1 (Kim et al. 2011; Lee et al. 2013; Manzano-Salgado et al. 2015), and single PFAA compounds vs. mixtures. However, possibly, also the potential influence of PFAAs on BW may differ between the maternal and fetal compartments.

Analyses of individual PFAAs suggested a tendency towards negative associations with BW and length in girls, and positive or null associations in boys. Differences in associations between prenatal exposures and BW by sex have been inconsistent in previous studies. Associations between BW and prenatal PFOS

Table 3. Estimated mean difference [95% confidence interval (CI)] in fetal growth indices in association with xenoestrogenic activities in serum PFAA fraction F3-W2 (categorical and continuous) in mother–newborn dyads from the Aarhus Birth Cohort, 2011–2013.

	Crude <i>n</i> = 694	Model 1 ^a <i>n</i> = 671	Model 2 ^b <i>n</i> = 670	Model 3 ^c <i>n</i> = 633
Birth weight (g)	—	—	—	—
XER ^d	—	—	—	—
53–105 (<i>n</i> = 173)	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>
105–124 (<i>n</i> = 175)	53 (53, 159)	56 (–51, 163)	28 (–59, 115)	33 (–63, 129)
124–146 (<i>n</i> = 172)	–16 (–128, 96)	–16 (–128, 96)	–28 (–116, 60)	–16 (–116, 85)
146–339 (<i>n</i> = 174)	–77 (–192, 37)	–83 (–196, 31)	–63 (–148, 22)	–53 (–153, 47)
IQR increase	–41 (–82, 0.4)	–48 (–90, –6)	–28 (–60, 4)	–32 (–60, 5)
XERcomp ^e	—	—	—	—
41–98 (<i>n</i> = 173)	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>
98.5–111 (<i>n</i> = 175)	–39 (–148, 70)	–34 (–146, 77)	–69 (–157, 18)	–82 (–173, 8)
111–132 (<i>n</i> = 172)	–28 (–136, 81)	–16 (–127, 94)	–60 (–144, 23)	–67 (–154, 20)
132.3–255 (<i>n</i> = 174)	0 (–109, 109)	–10 (–121, 102)	–33 (–121, 54)	–33 (–124, 58)
IQR increase	6 (–39, 50)	–2 (–48, 43)	–17 (–54, 20)	–15 (–57, 28)
W2-EEQ ^f	—	—	—	—
<LLOQ–0.07 (<i>n</i> = 278)	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>
0.07–1.5 (<i>n</i> = 73)	–43 (–171, 85)	–21 (–157, 115)	12 (–89, 113)	5 (–117, 127)
1.5–9.7 (<i>n</i> = 177)	–96 (–195, 4)	–90 (–190, 10)	–50 (–127, 26)	–57 (–146, 33)
9.7–150 (<i>n</i> = 174)	–92 (–190, 7)	–107 (–206, –8)	–79 (–157, –1)	–75 (–164, 13)
IQR increase	–21 (–42, –0.7)	–24 (–45, –2)	–15 (–31, 1)	–16 (–34, 2)
Birth length (cm)	—	—	—	—
XER ^d	—	—	—	—
53–105 (<i>n</i> = 173)	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>
105–124 (<i>n</i> = 174)	0.4 (–0.1, 0.9)	0.4 (–0.1, 0.9)	0.2 (–0.2, 0.7)	0.2 (–0.3, 0.7)
124–146 (<i>n</i> = 171)	0.2 (–0.4, 0.8)	0.2 (–0.4, 0.8)	0.1 (–0.4, 0.5)	0.1 (–0.4, 0.7)
146–339 (<i>n</i> = 175)	–0.6 (–1.2, 0.0)	–0.7 (–1.3, –0.1)	–0.6 (–1.1, –0.1)	–0.7 (–1.2, –0.1)
IQR increase	–0.3 (–0.5, 0.0)	–0.3 (–0.5, –0.1)	–0.2 (–0.4, 0.0)	–0.2 (–0.5, 0.0)
XERcomp ^e	—	—	—	—
41–98 (<i>n</i> = 173)	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>
98.5–111 (<i>n</i> = 174)	0.0 (–0.6, 0.6)	0.0 (–0.5, 0.6)	–0.1 (–0.6, 0.4)	0.0 (–0.6, 0.5)
111–132 (<i>n</i> = 171)	0.1 (–0.4, 0.7)	0.2 (–0.4, 0.7)	0.0 (–0.4, 0.4)	0.2 (–0.3, 0.6)
132.3–255 (<i>n</i> = 175)	–0.1 (–0.6, 0.5)	–0.1 (–0.6, 0.5)	–0.2 (–0.7, 0.3)	–0.2 (–0.7, 0.3)
IQR increase	0.0 (–0.2, 0.2)	0.0 (–0.2, 0.2)	–0.1 (–0.3, 0.1)	–0.1 (–0.3, 0.1)
W2-EEQ ^f	—	—	—	—
<LLOQ–0.07 (<i>n</i> = 278)	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>
0.07–1.5 (<i>n</i> = 73)	–0.1 (–0.7, 0.5)	0.0 (–0.6, 0.7)	0.2 (–0.3, 0.7)	0.2 (–0.4, 0.7)
1.5–9.7 (<i>n</i> = 177)	–0.6 (–1.1, 0.0)	–0.6 (–1.2, 0.0)	–0.4 (–0.9, 0.1)	–0.5 (–1.0, 0.1)
9.7–150 (<i>n</i> = 174)	–0.4 (–0.9, 0.1)	–0.5 (–0.9, 0.0)	–0.3 (–0.7, 0.1)	–0.4 (–0.8, 0.1)
IQR increase	–0.1 (–0.2, 0.0)	–0.1 (–0.2, 0.0)	–0.1 (–0.2, 0.0)	–0.1 (–0.2, 0.0)
Head circumference (cm)	—	—	—	—
XER ^d	—	—	—	—
53–105 (<i>n</i> = 171)	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>
105–124 (<i>n</i> = 173)	0.2 (–0.2, 0.5)	0.2 (–0.2, 0.5)	0.1 (–0.2, 0.4)	0.0 (–0.3, 0.4)
124–146 (<i>n</i> = 172)	0.1 (–0.2, 0.5)	0.2 (–0.2, 0.5)	0.1 (–0.2, 0.4)	0.1 (–0.2, 0.4)
146–339 (<i>n</i> = 174)	–0.1 (–0.5, 0.3)	–0.1 (–0.5, 0.3)	–0.1 (–0.4, 0.2)	–0.1 (–0.5, 0.2)
IQR increase	–0.1 (–0.2, 0.1)	–0.1 (–0.2, 0.0)	0.0 (–0.2, 0.1)	–0.1 (–0.2, 0.1)
XERcomp ^e	—	—	—	—
41–98 (<i>n</i> = 171)	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>
98.5–111 (<i>n</i> = 173)	0.0 (–0.4, 0.3)	0.0 (–0.4, 0.4)	–0.1 (–0.4, 0.2)	0.0 (–0.4, 0.3)
111–132 (<i>n</i> = 172)	0.0 (–0.4, 0.3)	0.0 (–0.3, 0.4)	0.0 (–0.3, 0.3)	0.0 (–0.3, 0.4)
132.3–255 (<i>n</i> = 174)	–0.1 (–0.3, 0.4)	0.0 (–0.3, 0.4)	0.0 (–0.3, 0.3)	0.0 (–0.4, 0.3)
IQR increase	0.0 (–0.1, 0.2)	0.0 (–0.1, 0.2)	0.0 (–0.2, 0.1)	0.0 (–0.2, 0.1)
W2-EEQ ^f	—	—	—	—
<LLOQ–0.07 (<i>n</i> = 278)	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>	<i>Ref</i>
0.07–1.5 (<i>n</i> = 73)	–0.1 (–0.6, 0.3)	–0.1 (–0.6, 0.4)	0.0 (–0.4, 0.3)	0.0 (–0.4, 0.4)
1.5–9.7 (<i>n</i> = 177)	–0.1 (–0.5, 0.2)	–0.1 (–0.4, 0.2)	0.0 (–0.3, 0.3)	–0.1 (–0.4, 0.2)
9.7–150 (<i>n</i> = 174)	–0.1 (–0.5, 0.2)	–0.2 (–0.5, 0.1)	–0.1 (–0.4, 0.2)	–0.1 (–0.4, 0.2)
IQR increase	0.0 (–0.1, 0.1)	0.0 (–0.1, 0.0)	0.0 (–0.1, 0.1)	0.0 (–0.1, 0.0)

Note: —, data not available; IQR, interquartile range; LLOQ, lower limit of quantification; *Ref*, reference; W2-EEQ, estrogen equivalence for the serum PFAA fraction F3-W2 alone; XER, xenoestrogenic receptor transactivation in the noncompetitive assay (serum PFAA fractions alone); XERcomp, xenoestrogenic receptor transactivation in the competitive assay (serum PFAA fractions + 24 pM E2).

^aModel 1 adjusted for age at delivery (continuous), prepregnancy body mass index (four categories), educational level (four categories), smoking (three categories), and alcohol intake (three categories).

^bModel 2 includes Model 1 covariates plus gestational age at birth (linear and quadratic).

^cModel 3 is restricted to term births (>37 gestational wk and 0 d) and is adjusted for Model 1 covariates.

^dXER categorized by quartiles (*n* = 173, 175, 172, and 174 for the lowest–highest quartiles in the full sample, respectively) or modeled as a continuous variable (IQR = 40.9).

^eXERcomp categorized by quartiles (*n* = 173, 175, 172, and 174 for the lowest–highest quartiles in the full sample, respectively) or modeled as a continuous variable (IQR = 33.7).

^fW2-EEQ categorized by quartiles (*n* = 278, 73, 177, and 174 for the lowest–highest quartiles in the full sample, respectively) or modeled as a continuous variable (IQR = 9.6 pM).

concentrations have tended to be null or positive in boys and negative in girls, while associations with prenatal PFOA have been negative, null, and positive in boys, and null or negative in girls

(Andersen et al. 2010; Bach et al. 2016; Washino et al. 2009). Bach et al. (2016) evaluated five additional PFAAs and reported negative (PFHpS) or null associations with BW in girls, and null

Table 4. Sex-stratified regression coefficients [95% confidence interval (CI)] and interaction *p*-values for indices of fetal growth per gender-specific interquartile range (IQR) increase in xenoestrogenic activities in the perfluorinated alkyl acid (PFAA) serum fraction F3-W2, Aarhus Birth Cohort, 2011–2013.

	Model 1 ^a			Model 2 ^b			Model 3 ^c		
	Boys <i>n</i> = 337	Girls <i>n</i> = 334	<i>p</i> -int	Boys <i>n</i> = 336	Girls <i>n</i> = 334	<i>p</i> -int	Boys <i>n</i> = 316	Girls <i>n</i> = 316	<i>p</i> -int
Birth weight (g)									
XER	–50 (–109, 9)	–39 (–97, 19)	0.80	–35 (–72, 2)	–21 (–73, 32)	0.67	–22 (–69, 24)	–36 (–92, 21)	0.72
XERcomp	–16 (–72, 39)	23 (–47, 93)	0.38	–40 (–83, 2)	13 (–47, 73)	0.15	–29 (–78, 20)	5 (–61, 72)	0.42
W2-EEQ (pM)	–18 (–39, 3)	–34 (–82, 15)	0.55	–16 (–33, 0)	–12 (–50, 26)	0.85	–14 (–32, 5)	–20 (–64, 25)	0.81
Birth length (cm)									
XER	–0.2 (–0.5, 0.1)	–0.4 (–0.7, 0.0)	0.49	–0.1 (–0.3, 0.1)	–0.3 (–0.6, 0.0)	0.44	–0.1 (–0.3, 0.2)	–0.4 (–0.7, –0.1)	0.14
XERcomp	0.0 (–0.3, 0.3)	0.0 (–0.4, 0.3)	0.92	–0.1 (–0.3, 0.1)	–0.1 (–0.4, 0.2)	0.94	–0.1 (–0.3, 0.2)	–0.2 (–0.5, 0.2)	0.56
W2-EEQ (pM)	–0.1 (–0.2, 0.0)	–0.2 (–0.4, 0.0)	0.25	–0.1 (–0.2, 0.0)	–0.1 (–0.3, 0.1)	0.78	0.0 (–0.1, 0.0)	–0.2 (–0.4, 0.0)	0.17
Head circumference (cm)									
XER	–0.1 (–0.3, 0.1)	–0.1 (–0.3, 0.1)	0.67	0.0 (–0.1, 0.1)	–0.1 (–0.3, 0.1)	0.44	0.0 (–0.1, 0.2)	–0.1 (–0.3, 0.0)	0.21
XERcomp	0.0 (–0.1, 0.2)	0.0 (–0.2, 0.2)	0.83	0.0 (–0.2, 0.2)	0.0 (–0.3, 0.2)	0.79	0.0 (–0.1, 0.2)	–0.1 (–0.3, 0.1)	0.45
W2-EEQ (pM)	0.0 (–0.1, 0.1)	–0.1 (–0.3, 0.0)	0.19	0.0 (–0.1, 0.1)	–0.1 (–0.2, 0.1)	0.35	0.0 (–0.1, 0.1)	–0.1 (–0.3, 0.1)	0.08

Note: —, data not available; Estimates stratified by sex were derived using separate models for boys and girls. Interaction *p*-values are *p*-values for product interaction terms between each exposure variable and sex from separate models. IQRs of XER were 42.3 and 38.6 for boys and girls, respectively. IQRs of XERcomp were 33.3 and 33.9 for boys and girls, respectively; IQRs of EEQ were 8.9 and 11.0 pM for boys and girls, respectively. IQR, interquartile range; *p*-int, interaction *p*-value, was derived by using interaction models in the multivariable linear regressions; W2-EEQ, estrogen equivalence quotient for the F3-W2 fraction; XER, xenoestrogenic receptor transactivation in the noncompetitive assay (serum PFSA fractions alone); XERcomp, xenoestrogenic receptor transactivation in the competitive assay (serum PFSA fractions + 24 pM E2).

^aModel 1 adjusted for age at delivery (continuous), prepregnancy body mass index (four categories), educational level (four categories), smoking (three categories), and alcohol intake (three categories).

^bModel 2 includes Model 1 covariates plus gestational age at birth (linear and quadratic).

^cModel 3 is restricted to term births (>37 gestational wk and 0 d) and is adjusted for Model 1 covariates.

(PFHpS, PFUnA), negative (PFHxS), or positive (PFOA, PFNA, PFDA) in boys. Robledo et al. (2015) reported that the association between BW and prenatal maternal serum PFOSA was negative in boys and close to the null in girls, but sex-specific estimates for six additional PFAAs were not reported. We did not model associations with PFOSA in our study population because all samples had concentrations <LOQ. In contrast with the patterns of associations by sex for individual PFAAs in our study population, there was little evidence of sex-specific patterns for associations with measures of combined PFSA-induced xenoestrogenic activities.

To our knowledge, we are the very first developing the method to extract PFAAs from human serum while simultaneously removing endogenous hormones (Bjerregaard-Olesen et al. 2015). We have earlier developed a method for serum extraction of the lipophilic persistent organic pollutants (lipPOP), such as polychlorinated biphenyls and organochlorine pesticides free of endogenous hormones for measurement of the combined lipPOP-induced xenoestrogenicity and xenoandrogenicity (Bonefeld-Jørgensen et al. 2006; Hjelmborg et al. 2006; Krüger et al. 2012). In Inuit, we found an inverse association between the xenohormone transactivity and lipPOP serum concentrations using the MVLN cell line, as in the present study, for xenoestrogen determination (Krüger et al. 2012). In Olea's lab, they as well developed a method for measuring the total xenoestrogenicity (TEXB- α) for extraction of serum, human adipose, and placenta (Arrebola et al. 2012; Fernandez et al. 2007; Sonnenschein et al. 1995; Vilahur et al. 2013). Although the extraction methods for TEXB- α is not identical, the methods are very similar to our SPE-HPLC extraction of total serum lipPOP mixture. However, to determine the lipPOP-induced xenoestrogenic activity, the Olea lab uses the E-screen MCF-7 proliferative bioassay. In an earlier study on pesticides, we demonstrated high similarity between xenoestrogenicity determined by the E-screen and ER transactivity (Andersen et al. 2002). However, data for some pesticides differed considerably. Using the TEXB- α –E-screen method, Olea's lab reported a male-specific positive association between xenoestrogen levels in placenta and BW (Vilahur et al. 2013).

Other studies have reported that higher maternal testosterone and higher maternal free thyroxine (fT4) levels in early pregnancy,

as well as lower cord blood fT4 levels, were associated with lower BW (Carlsen et al. 2006; Medici et al. 2013). We have previously reported that PFAAs antagonized the dihydrotestosterone-induced androgen receptor (AR) *in vitro* in CHO-K1 cells (Kjeldsen and Bonefeld-Jørgensen 2013) and inhibited the function of the thyroid hormone (TH) system in GH3 cells (Long et al. 2013). In addition, PFOS and PFOA were reported to agonize the human peroxisome proliferator-activated receptor (PPAR) isoforms α and γ *in vitro* in 3T3-L1 cells (Vanden Heuvel et al. 2006), and PFOS up-regulated the PPAR γ gene *in vitro* in a rodent neural stem cell culture and *in vivo* in cortical tissues from neonatal mice (Wan Ibrahim et al. 2013). Placental PPAR γ expression was associated with higher BW in 116 newborns from Spain (Díaz et al. 2012). Thus, associations between PFAAs and fetal growth might also be mediated through disruption of the AR, TH, and PPAR systems.

To our knowledge, there are no prior reports on predictors of PFSA-induced activities, and therefore, the adjustment variables in the present study were *a priori* selected from predictors of PFSA concentrations. Hence, we adjusted for the covariates maternal age, prepregnancy BMI, educational level, smoking, and alcohol intake in the primary analysis (Bjerregaard-Olesen et al. 2016a). In addition, we included only nulliparous women to prevent confounding by parity (Bach et al. 2016). Moreover, we conducted sensitivity analyses to investigate the pathways of the associations. Firstly, we included gestational duration as an additional adjustment variable. Gestational duration was not included in the primary analysis, since most studies, including the present (data not shown), found no significant associations between PFAAs and gestational duration (Bach et al. 2015). However, in our sensitivity analyses, when we additionally adjusted for gestational duration, we found that most of the associations between the activity variables and pregnancy outcomes were weaker, except for XERcomp vs. BW. We did not adjust models of associations with BW for birth length, which might act as a causal intermediate if PFAAs influence BW through effects on length. In addition, adjusting for birth length could introduce collider bias if length and weight are influenced by shared unmeasured factors. The pregnancy outcomes were recorded and validated by trained health care professionals. We expect little measurement error with regard to BW, whereas some uncertainty is expected on the

measures of birth length and head circumference (Bach et al. 2016). As discussed previously (Bach et al. 2016), the selection of only women with live-born offspring may have introduced selection bias, although this would require a strong association between PFAA exposure and risk of miscarriage. For the present study, we included 702 of the previously selected 1,507 pregnant women. The 702 women included in the present study, which was limited to women enrolled in 2011–2013, had a lower median concentration of summed PFAAs (12.7 ng/mL) than the total population of 1,507 women included in our previous study (14.3 ng/mL) (Bach et al. 2016). This is consistent with the downward trend in PFAAs from 2008 to 2013 among participants in the ABC cohort (Bjerregaard-Olesen et al. 2016b). Women included in the present study also were more likely to have never consumed alcohol than women in the larger population (43 vs. 33%). Recently, we reported that serum concentrations of individual PFAAs differed substantially between pregnant women from Denmark, Norway, Greenland, and China (Bjerregaard-Olesen et al. 2016c). The PCA analysis suggests that PFAA-induced xenoestrogenic activities may depend on the composition of the PFAA mixture, and hence, associations estimated for the present study might not be generalizable to populations with different PFAA exposure profiles.

Specific single-nucleotide polymorphisms (SNPs) in ER genes might affect the ER activity (Sundermann et al. 2010). We are not aware of the SNPs in the ABC women or whether these SNPs affect the functionality of the ER. However, it is possible that the xenoestrogenic activity measured in the MVLN cell line does not reflect the activity that would actually be elicited by the ER in the involved ABC women.

The ER transactivation of each sample was analyzed in triplicate to reduce measurement error, and for most samples, there was less than 10% variation between the measurements. The samples were related to a procedural blank (extracted double-distilled water) to reduce the potential influence of contamination during the extractions. In addition, we used a pooled serum control (KHK) to ensure comparability between the assays. The W2-EEQ is the calculated E2 equivalent for the ER transactivation induced by the serum PFAA extract fraction F3-W2. Thus, it is a comparison of the serum PFAA-induced xenoestrogenicity with the E2-induced estrogenicity. The W2-EEQ was derived from the XER, but was calculated using sigmoidal E2 dose–response curves for each batch of samples to correct for potential day-to-day variation in the biological assays (Rajapakse et al. 2004). Despite these differences, both XER and W2-EEQ were inversely associated with BW and birth length, suggesting that the PFAAs may have a negative impact on human fetal growth. The PFAA fractions (F3-W2) might contain trace amounts of the sulfated steroid hormones dehydroepiandrosterone sulfate and estrone 3-sulfate as well as other unidentified anionic compounds (Bjerregaard-Olesen et al. 2016d, 2016e). However, the tests in our previous studies showed no interference from these sulfated steroids (Bjerregaard-Olesen et al. 2016d, 2016e), and hence, we do not expect any interference in the present study. We included only serum samples taken in early pregnancy (gestational wk 11–13; Figure 1) to avoid any potential interference from high serum levels of endogenous estrogens as expected in late pregnancy.

Conclusions

We studied associations between birth outcomes and measures of the net xenoestrogenic activity of maternal serum PFAA mixtures, in addition to associations with individual serum PFAAs, and found that higher PFAA-induced xenoestrogenic activity was associated with lower BW and length. However, further studies

are needed to confirm our findings and evaluate the potential contribution of other biological pathways to associations between prenatal PFAA exposures and birth outcomes, including the AR, PPAR, and TH systems.

Acknowledgments

We thank our fellow researchers of the FETOTOX project (<http://www.fetotox.au.dk>) for their contributions to the work, especially technical assistant D. A. Dang for carrying out many of the ER transactivation assays and M. Ghisari for assisting with the serum extractions. We thank M. Pons providing the MVLN cell line.

The Danish Council supported the study for Strategic Research (Grant 10-092818). The funders were not involved in the research activities. The Aarhus Birth Cohort Biobank is funded by a grant from the Danish National Research Foundation with additional support from TrygFonden and the Aarhus University Research Foundation.

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